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Preclinical studies of signaling pathways in a mutant mouse model of hormone-refractory prostate cancer

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14. ABSTRACT We have been investigating targeted therapies for the treatment of advanced prostate cancer using a genetically-engineered mouse model of the disease. We performed pre-clinical studies to examine the consequences of combinatorial inhibition of these signaling pathways using Rapamycin, an inhibitor of mTOR, and PD0325901, a MEK inhibitor, is potently anti-tumorigenic in mouse models of castration-resistant prostate cancer. Current studies reported herein demonstrate that this combination is also effective for tumor suppression of advanced prostate cancer models and can improve survival. Studies in progress are evaluating new combinations and also evaluating molecular changes in response to therapy.

15. SUBJECT TERMS
Rapamycin, mTOR, PD0325901, MEK inhibitor, Nkx3.1, Pten, Akt/mTOR, Erk Map

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1. Introduction

The prostate is critically dependent on androgen receptor signaling for all stages of its normal growth and development as well as all stages of cancer. Accordingly, androgen-deprivation has been widely used for the treatment of prostate cancer. Although this therapy initially results in tumor regression and decreased PSA levels, most patients develop hormone-refractory tumors that are resistant to available treatments. Counter-intuitively, most hormone-refractory tumors remain dependent on androgen receptor (AR) signaling and have devised mechanisms to bypass the need for testicular androgens for tumor growth. Thus, it is essential to understand how these tumors arise in the absence of testicular androgens and to use this understanding to develop strategies to control the development of hormone-refractory disease. Our proposal is based on published studies from us and others showing that two key signaling pathways, namely the Akt/mTOR and B-Raf/Erk MAP kinase pathways, promote hormone-refractory prostate cancer in an AR-dependent manner. We **hypothesize** that combination therapy for the PI3Kinase/Akt/mTOR and B-Raf/MEK/Erk MAP kinase pathways will be effective for treating or preventing hormone-refractory prostate cancer.

We had proposed to: (1) **Targeting the Akt/mTOR and B-Raf/Erk MAP kinase pathways —** investigate the consequences of combinatorial inhibition of these signaling pathways at distinct steps in cell culture and in a novel human organ culture assay using available pharmacological agents. (2) **Inhibition of Akt/mTOR and B-Raf/Erk MAP kinase pathways in pre-clinical studies —** evaluate the consequences of inhibiting Akt/mTOR and B-Raf/MAP signaling following androgen-deprivation in pre-clinical studies in a mouse model of hormone-refractory prostate cancer. These pre-clinical studies will test the **hypothesis** that pharmacological manipulation of the Akt/mTOR and B-Raf/MAP kinase pathways will control or prevent hormone-refractory disease.

2. Body

2.A. Background and rationale (summary of published work and findings previously reported)

Because of its central relevance for prognosis of patients with prostate cancer, we have focused on investigating the molecular mechanisms underlying castration-resistant disease and the identification of new molecular targets for therapeutic intervention. In comparative analyses of prostate cancer progression in intact versus castrated *Nkx3.1; Pten* mutant mice, we found that the Akt/mTOR and Erk MAP kinase signaling pathways are coordinately up-regulated during cancer progression, particularly in androgen-independent tumors (1). Furthermore, we showed that these two signaling pathways cooperate to promote androgen-independent tumor growth *in vivo*, consistent with studies in human prostate cancer cells (2).

Based on these observations, we performed **pre-clinical studies** to test whether combinatorial inhibition of these pathways could block cancer progression, in androgen-independent contexts (3). For these studies, we used rapamycin to target the Akt/mTOR signaling pathway and a Pfizer MEK inhibitor (PD035901) to target MEK/MAP kinase signaling, and we examined pS6 and pErk respectively as downstream read-outs of pathway activity. Using our CASP cells, we found that these agents act cooperatively to promote cell toxicity, mediated in part by the pro-apoptotic regulator, Bim.

We next performed preclinical studies to evaluate the efficacy of this combination *in vivo*. To do so, we treated the *Nkx3.1; Pten* mutant mouse model with combination therapy using rapamycin and PD035901 profoundly affected the growth of androgen-independent prostate tumors. In particular, delivery of these agents in combination (but not individually) for a period of one month resulted in a significant reduction in the occurrence of PIN/cancer lesions, as well as a 2.5 fold reduction in tumor size and 14 fold decrease in proliferation. Furthermore, analyses of human tissue microrarrays revealed that 25% of human prostate cancer cases display activation of both Akt/mTOR and MEK/MAPK pathways (3). These findings suggest that a sizable population of prostate cancer patients may benefit from combination treatment targeting these two signaling pathways. In ongoing studies, we are pursuing pre-clinical studies in this mouse model to evaluate the best combination(s) of pathway-blocking agents, which should ultimately guide the development of new clinical trials for patients with castration-resistant prostate cancer.

2.B. Summary of progress and plans for coming year

Based on the preceding studies, we have subsequently focused on: (1) testing this combination therapy in prostate cancer mouse models that display more aggressive disease to evaluate their efficacy for survival and metastases; (2) performing gene expression profiling to evaluate the response to treatment; and (3)

evaluated newer agents that are more likely to be available in the clinic. All of these studies are in progress at the time of this report and our findings thus far are described below.

Advances in prostate cancer mouse model: We have now developed more advanced prostate cancer mouse models based on conditional loss-of-function of *Pten* (to activate the mTOR signaling pathway) and conditional activation of *Kras* and *Braf* (to activate the MEK/Map Kinase signaling pathway), which display more aggressive prostate tumors as well as metastases. These models are based on a Tamoxefin-inducible Cre allele ($Nkx3.1^{CreERT2}$), which was made in Michael Shen's laboratory and has been recently reported (4). This $Nkx3.1^{CreERT2}$ allele was crossed with a *Pten* conditional allele (obtained from the NCI Mouse Repository) alone or in combination with a conditionally activatable Kras or B-Raf allele (obtained from the NCI Mouse Repository or collaboratively from Martin McMahon (UCSF), respectively). The phenotypes of these mice are summarized in the table below and their survival spectra in Fig. 1.

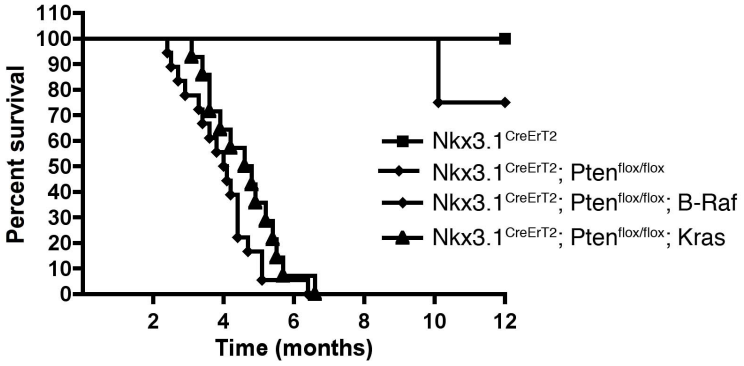

Figure 1. Survival curves following inducible deletion. Mice of the indicated genotypes were induced with tamoxifen and monitored for survival for the indicated period.

Table 1. Summary of Prostate Cancer Phenotype

Experimental Group	Induction	Age	*n*	Phenotype
Group I. Androgen-dependent mice lacking *Pten*				
$Nkx3.1^{CreERT2/+}$	Induced/ Uninduced	Up to 20 months	8	Prostate histology within normal limits
$Nkx3.1^{CreERT2/+}; Pten^{flox/flox}$ or $Nkx3.1^{CreERT2/+}; Pten^{flox/flox}; LucRep$	Uninduced	<18 months	17	Prostate histology within normal limits
	Induced	<6 months	20	High-grade PIN with invasion
	Induced	6-18 months	11	Adenocarcinoma with rare metastases to distant organs; oldest mice display poorly differentiated tumors
Group II. Androgen-independent mice lacking *Pten*				
$Nkx3.1^{CreERT2/+}; Pten^{flox/flox}$ or $Nkx3.1^{CreERT2/+}; Pten^{flox/flox}; LucRep$	Uninduced	<18 months	8	Regressed prostate; histology within normal limits
	Induced	<16 months	15	High-grade PIN and/or adenocarcinoma with rare metastases to distant organs; oldest mice display poorly differentiated tumors
Group III. Mice lacking *Pten* with activated Kras (androgen-dependent and androgen-independent)				
$Nkx3.1^{CreERT2/+}; Pten^{flox/flox}; Kras^{LSL/+}$ or $Nkx3.1^{CreERT2/+}; Pten^{flox/flox}; Kras^{LSL/+}; LucRep$	Uninduced	<6 months	10	Prostate histology within normal limits
	Induced	<7 months	26	Highly aggressive and poorly differentiated tumors with frequent metastases to lymph nodes and distant organs
Group IV. Mice lacking *Pten* with activated B-Raf (androgen-dependent and androgen-independent)				
$Nkx3.1^{CreERT2/+}; Pten^{flox/flox}; B\text{-}Raf^{LSL/+}$ or $Nkx3.1^{CreERT2/+}; Pten^{flox/flox}; B\text{-}Raf^{LSL/+}; LucRep$	Uninduced	<6 months	11	Prostate histology within normal limits
	Induced	<7 months	36	Highly aggressive and poorly differentiated tumors with frequent metastases to lymph nodes and distant organs

We are now using these advanced prostate cancer mouse models to investigate the efficacy of the Rapamycin and PD035901 combination therapy for survival and suppression of metastases, as well as suppression of tumor growth. In our findings thus far, we have found that the Rapamycin and PD035901 combination therapy works effectively in these more advanced models of prostate cancer. For example, in the $Nkx3.1^{CreERT2/+}; Pten^{flox/flox}; Kras^{LSL/+}$ model, treatment for one month Rapamycin and PD035901 combination therapy significantly reduced tumor size and also significantly improved survival in these mice (Fig. 2).

New progress in molecular analyses and evaluating new combination therapy: Given the success of our approach, we are now focusing on elucidating the genes responsible for the observed phenotypic changes in response to combination therapy in the mouse models. For this purpose, we have performed gene expression profiling analyses to compare the gene expression profiles in the vehicle treated, combination treated or "survivor" mice. These experiments have just been initiated and we have no findings to report.

In addition, we have also been investing new combinations that target the Akt/mTOR signaling pathway and also would be potential therapies that could be translated to the clinic. Among the drugs that we have been testing are two that we have recently obtained from Merck – namely an Akt inhibitor (MK-2206) and a new mTOR inhibitor (MK-8669). We have now just begun testing these agents singlely and in combination in our mouse models. The initial results look very promising but the cohort size is too small to make definitive assessment of suitability; additional studies are ongoing.

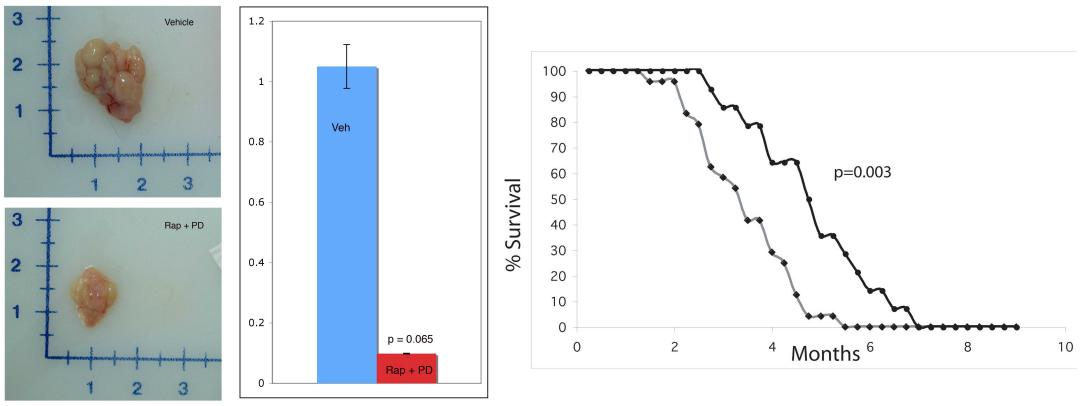

Figure 2. Treatment with combination therapy suppresses tumors and improves survival of in *Nkx3.1*$^{CreERT2/+}$; *Pten*$^{flox/flox}$; *Kras*$^{LSL/+}$ mice. Mice (20 per group) were provided vehicle or combination therapy and sacrificed to assess tumor weight (*left)* or monitored for survival for the indicated period (*right*).

2.C. Relevance of major findings to Statement of Work

Aim 1: Targeting the Akt/mTOR and B-Raf/MAP kinase pathways:

Task 1: Testing relevant drugs in cell culture for optimal dosage and for cytotoxicity (Months 1-9).

As a requisite first step, we will obtain pharmacological inhibitors to the various components of the Akt and Erk MAP kinase signaling pathways and test their optimal dosage in cell culture. These studies will use mouse and human prostate cancer cell lines including androgen-dependent and androgen-independent cell lines (e.g., human — LNCaP, PC3, VCAP; mouse — CASP2.1, CASP1.1).

Progress this year and plans for coming year:

These studies are now completed for Rapamycin and PD035901 and have been initiated for the new combination agents obtained from Merck, as described above namely an Akt inhibitor (MK-2206) and a new mTOR inhibitor (MK-8669).

Task 2: Test the drugs individually and in combination for ability to inhibit androgen independence in cell culture (Months 4-12)

Progress this year and plans for coming year:

These studies are now completed for Rapamycin and PD035901 and have been initiated for the new combination agents obtained from Merck, as described above namely an Akt inhibitor (MK-2206) and a new mTOR inhibitor (MK-8669).

Task 3: Test the drugs individually and in combination using human organ culture (Months 12-24)

Progress this year and plans for coming year:

We have had difficulty obtaining prostate tissues in sufficient amounts for organ culture study, so we have now shifted our focus to using xenograft models; we are just about to initiate this work.

Aim 2: Inhibition of Akt/mTOR and B-Raf/MAP kinase pathways in pre-clinical studies:

Task 1: Testing relevant drug combinations for optimal dosage and PD *in vivo* (Months 12-18).

Focusing on the most promising combinations of drugs identified in the studies in Aim 1, we will first investigate the optimal dosage individually and in combination in vivo. Our starting point will be the recommended dosages from the published literature (see Table above). We will also determine the efficacy of the agents in inhibiting their targets in vivo (PD studies).

Progress and plans:

These studies are now completed for Rapamycin and PD035901 and have been initiated for the new combination agents obtained from Merck, as described above namely an Akt inhibitor (MK-2206) and a new mTOR inhibitor (MK-8669).

Task 2: Test the drugs individually and in combination for ability to inhibit androgen independence *in vivo* (Months 18-36).

Progress and plans:

These studies are now completed for Rapamycin and PD035901 in the original *Nkx3.1Pten* model. They are now in progress in our new models of advanced prostate cancer (as described above) and have been initiated for the new combination agents obtained from Merck, as described above namely an Akt inhibitor (MK-2206) and a new mTOR inhibitor (MK-8669).

3. Key Research Accomplishments

- Showed that combination therapy with Rapaymcin and PD035901 acts synergistically in cell culture by regulating Bim1
- Completed studies in the *Nkx3.1; Pten* mutant mouse model of prostate cancer to evaluate the PK/PD of Rapaymcin and PD035901 *in vivo*
- Completed preclinical studies in the *Nkx3.1; Pten* mutant mouse model of prostate cancer to examine the efficacy of this combination for treatment of androgen-independent prostate cancer.
- Obtained additional agents from Merck for testing efficacy in cell culture and *in vivo*.
- Performed initial studies in our new conditional models of advanced prostate cancer to evaluate the PK/PD of Rapaymcin and PD035901 *in vivo* for survival and suppression of metastases
- Initiated studies to evaluate the molecular response to these agents *in vivo*

4. Reportable Outcomes

- New mouse models of advanced prostate cancer and metastases
- New preclinical studies that can potentially be translated to the clinic

5. Conclusion

Although most men diagnosed with early stage prostate cancer have favorable outcomes, those with advanced disease and particularly hormone-refractory prostate cancer eventually succumb to lethality since treatment options are limited. We have been investigating targeted therapies for advanced prostate cancer using genetically-engineered mouse models of the disease. Based on previous studies showing that the Akt/mTOR and Erk MAP kinase signaling pathways cooperate in prostate cancer progression, we performed pre-clinical studies to examine the consequences of combinatorial inhibition of these signaling pathways for prostate tumorigenesis in androgen-dependent and -independent contexts. We found that combination therapy using Rapamycin, an inhibitor of mTOR, and PD0325901, a MEK inhibitor, is potently anti-tumorigenic in castration-resistant prostate cancer. We are now expanding these findings to evaluate the consequences for survival and metastases, to evaluate additional combinations that target these pathways, and to elucidate molecular pathways that are responsible for these drug responses *in vivo*.

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